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Double Label Immunohistochemistry For The Separate Observation Of Two Antigens Using Epipolarization Microscopy For The Immunogold-Silver Technique And Fluorescence Microscopy For The Alkaline Phosphatase Staining

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Introduction

For immunohistochemical double staining basically two approaches exist, either double-immunofluorescence techniques or double-immunoenzyme detection methods. Both approaches, however, have disadvantages.
(i) The fading of the fluorescence signal at excitation and during storage.
(ii) Thus far it has been proven impossible in immunoenzyme double staining methods to examine one enzymatic reaction product, singling out the other precipitate, as can be done with two fluorochromes. Despite the fact that conditions can be created to optimize the contrast between the two basic colours and the mixed-colour at sites of co-localization (Van der Loos et al., 1993), specimens cannot be examined or photographically recorded for their individual colours, not even with the aid of monochromatic filters. Most successful in this respect, but still not ideal, is the combination of an immunoenzyme method with the immunogold-silver (IGS) technique. This approach offers the possibility to observe both enzymatic reaction products and silver precipitate with bright-field microscopy, while the silver precipitate can be visualized also with epipolarization microscopy. The latter technique will not reveal the enzymatic reaction product. An obvious disadvantage is the high costs involved with full-colour reproduction.
(iii) The localization of enzymatic reaction products usually is more diffuse and less well defined compared to the staining accuracy of fluorochromes or that of a silver precipitate (Van der Loos, personal observation).

What's New?

Alkaline phosphatase (AP) reaction products, using Fast Red TR as chromogen, show unique fluorescent properties. Minute amounts of the reaction product produce an intense red fluorescence signal with hardly any fading when excited by green light (Murdoch et al., 1990; Speel et al., 1992).
Since it is well known that a silver precipitate can be studied with epipolarization microscopy, the idea was born to combine the fluorescent AP method with the IGS technique. It appeared, however, that Fast Red TR also produced a strong epipolarization image. Therefore we searched for a different chromogen. The ideal conditions were fulfilled completely by the Becton Dickinson CAS Red AP activity visualization kit (Van der Loos and Becker, 1994). Later, the Vector Red kit (Vector Laboratories) for AP activity detection, showed identical characteristics (van der Loos, personal observation). This set-up opened the possibility to perform an immunohistochemical double staining procedure, combining fluorescence (AP) with epipolarization (IGS) microscopy for the detection of the two reaction products in one and the same section.

The Protocol

Antisera, antibody/enzyme conjugates and streptavidin reagents were diluted in Tris/HCl (50 mM, pH 7.8) buffered saline (TBS) + 1% Bovine Serum Albumin (BSA).
TBS washings were performed between all steps (3 x 2 min) and all incubations were performed at room temperature.
Antibody/Ultra Small Gold (USG) conjugates were diluted in TBS + 0.8% BSA + 0.1% cold water fish skin gelatine. This solution was also used for washing prior to and after the incubation with the antibody/USG conjugate (3 x 10 min).

1. Complete an appropriate double staining protocol (van der Loos et al., 1993) on either acetone fixed cryostat sections, cytospins, cell cultures, imprints etc. or routinely formalin fixed and paraffin embedded sections. The double staining protocol requires the use of an alkaline phosphatase (AP-)conjugated reagent for one antigen and an Ultra Small Gold (USG-) labeled reagent for the other antigen.
2. Final washing with saline buffered with Tris/HCl (50 mM, pH 7.8).
3. AP activity is detected using the CAS Red kit (Beckton Dickinson) or Vector Red kit according to the manufacturer's instructions.

1µl of 1 M Levamisole is added for inhibition of endogenous AP activity (Borgers, 1973). The total solution is cleared through a Milex-AA 0.8 µm filter and directly brought on the specimens. The formation of the AP reaction product is checked with bright-field microscopy at a low magnification. When a pink/red reaction product becomes visible, the reaction is stopped in running tap water. This reaction should be preferably completed within 5 min at room temperature, in order to keep the formation of non-specific background staining at a minimal level.

4. Wash specimen in running tap water for 10 min and immerse in distilled water.
5. Perform silver enhancement using the freshly prepared Aurion R-gent reagent. The reaction is checked with bright-field microscopy at low magnification. When a weak brownish precipitate becomes visible (5-20 min), silver enhancement is stopped by immersing the specimens in distilled water.
6. Wash with running tap water (10 min).
7. Optional: short nuclear counterstain with haematoxilin, followed by running tap water.
8. Immerse specimens in distilled water and mount with Glycergel (DAKO).
9. Observe IGS reaction product with epipolarization filter and AP reaction product with standard rhodamine filter set. Reduce excitation light as much as possible with a grey filter.
10. Photographic recording in black-and-white using Ilford HP5 (routinely processed) or in colour using Kodak EL 400 (routinely processed). For both 400 ASA film types the epipolarization and fluorescence images are individually exposed automatically at 1600-3200 ASA. For double exposure using Kodak EL 400, the images are individually exposed automatically at 3200 ASA.

Evaluation/Discussion

This Newsflyer presents a new method for the detection of two antigens in one section. The technique is based on the combination of a fluorescent AP activity detection method and the IGS technique. The AP reaction product shows an intense red fluorescence on green light excitation, but no signal with epipolarization illumination. The IGS technique produces a distinct signal with epipolarization, but no fluorescence signal. This allows individual examination and photographic recording of two antigens, even with black-and-white photography (Van der Loos and Becker, 1994). The stability of the fluorescent AP reaction product appeared to be superior compared with traditional fluorochromes. At the time of writing this Newsflyer, our oldest specimens have been stored at room temperature for 24 months and still exhibit a bright fluorescence. Fading of the fluorescence image with high intensity excitation light is reduced using a grey filter. The stability of the IGS reaction product (a silver precipitate) during storage at room temperature or at epi-illumination with polarized UV light is beyond dispute. Specimens, which underwent single IGS staining mounted either with Glycergel (DAKO) or an organic medium, are stored for 5 years or longer without loosing their original epipolarization image (Van der Loos, personal observation).

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A Comparison Between The Streptavidin-Biotin-Alkaline Phosphatase Method And The Immunogold Technique Using Ultra Small Gold Particles And Silver Enhancement

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Introduction

Fragile X syndrome is the most common basis of inherited mental retardation and is caused by a mutation in the FMR1 gene which involves an increase in length of a stretch of CGG repeats just upstream of the coding region. In the normal population the number of CGG repeats varies between 6-50 repeats. CGG repeat numbers between 50 and 200 (premutation) are observed in unaffected carrier males and females. In patients with fragile X syndrome this repeat shows an expansion of more than 200 repeats (full mutation), associated in all males with mental retardation. Due to this large number of repeats, transcription suppression through methylation of the promotor region of the FMR1 gene occurs, resulting in absence of the encoded protein FMRP. This lack of FMRP protein is responsible for the fragile X phenotype. Characterization of the gene defect of fragile X syndrome led to improved molecular diagnosis of the syndrome via Southern blot analysis and a PCR test. Recently, an immunocytochemical test at the light microscopic level on blood smears is described to identify fragile X patients. This immunocytochemical test is based on the presence of FMRP protein in normal lymphocytes and the absence of FMRP protein in lymphocytes from fragile X patients. The FMRP protein was visualized in blood smears by a three step immunoincubation procedure using a streptavidin-biotin-alkaline phosphatase complex. This presentation deals with the comparison of the alkaline phosphatase labelling technique and the immunogold method using ultra small gold particles and silver enhancement with Aurion R- Gent.

Methods

Fixation and Permeabilization

Blood smears were made from one drop of blood immediately after bleeding. Slides were airdried and either stored at room temperature (max. 3 weeks) or directly fixed in 0.1 M phosphate buffer (Sörrensen, pH 7.3), containing 3% paraformaldehyde for 10 minutes at room temperature. Cells were permeabilized by treatment with 100 % methanol for 20 minutes at room temperature. Subsequently, slides were washed in PBS (10 mM Phosphate buffer, 150 mM NaCl) +0.1% BSA-c for 10 minutes.

Immunoincubation

Immunoincubation was performed using the Shandon disposable coverplate incubation system. Slides were washed 2X10 minutes with PBS, 0.1% BSA-c.

Alkaline Phosphatase Labelling (3 Step Immunoincubation)

1. Incubation with mouse monoclonal antibodies 1A1 against FMRP protein (1:1200 with PBS, 0.1% BSA-c) for 16 hours at 4°C followed by 3X5 minutes washing with PBS, 0.1% BSA-c.
2. Incubation with goat anti-mouse immunoglobulins conjugated with biotin for 60 minutes (1:200 with PBS, 0.1% BSA-c) at room temperature followed by 3X5 minutes washing with PBS, 0.1% BSA-c.
3. Incubation with streptavidin-biotinylated alkaline phosphatase complex for 45 minutes at room temperature (1:100 with PBS, 0.1% BSA-c) followed by 3X10 minutes washing with PBS, 0.1% BSA-c.
4. Wash in 0.1 M TRIS for 5 minutes.
5. New Fuchsin substrate-chromogen system for 25 minutes at room temperature. Levamisole is added in the substrate solution to block endogenous alkaline phosphatase activity.
6. Wash in 0.1 M TRIS for 5 minutes.
7. New Fuchsin substrate-chromogen system for 25 minutes at room temperature.
8. Wash in aqua dest for 5 minutes.
9. Slides are counter stained with Gill's haematoxylin for 2 seconds and mounted with aquamount.

Immunogold Labelling (2 Step Immunoincubation)

1. Incubation with mouse monoclonal antibodies 1A1 against FMRP protein (1:4000 with PBS, 0.1% BSA-c) for 16 hours at 4°C followed by 3X5 minutes washing with PBS, 0.1% BSA-c.
2. Incubation with goat anti mouse immunoglobulins conjugated with ultra small gold particles for 60 minutes at room temperature (1:80 with PBS, 0.1% BSA-c) followed by 3X10 minutes washing with PBS, 0.1% BSA-c.
3. Wash in PBS for 10 minutes.
4. Postfixation with PBS, containing 2% glutaraldehyde for 10 minutes.
5. Wash 3X10 minutes in PBS.
6. Wash 3X10 minutes in aqua dest.
7. Silver enhancement with Aurion R-Gent for 20 minutes at room temperature.
8. Wash 2X5 minutes in aqua dest.
9. Silver enhancement with Aurion R-Gent for 20 minutes at room temperature.
10. Wash 2X5 minutes in aqua dest.
11. Slides were counter stained in Nuclear Fast Red for 45 seconds, dehydrated in ethanol/xylene and mounted with Pertex.

Immunogold Labelling (3 Step Immunoincubation)

1. Incubation with mouse monoclonal antibodies 1A1 against FMRP protein (1:4000 with PBS, 0.1% BSA-c) for 16 hours at 4°C followed by 3X5 minutes washing with PBS, 0.1% BSA-c.
2. Incubation with goat anti-mouse immunoglobulins conjugated with biotin (1:200 with PBS, 0.1% BSA-c) for 60 minutes at room temperature followed by 3X5 minutes washing with PBS, 0.1% BSA-c.
3. Incubation with goat anti-biotin conjugated with ultra small gold particles for 60 minutes at room temperature (1:30 with PBS, 0.1% BSA-c) followed by 3X10 minutes washing with PBS, 0.1% BSA-c.
4. Wash in PBS for 10 minutes.
5. Postfixation with PBS, containing 2% glutaraldehyde for 10 minutes.
6. Wash 3X10 minutes in PBS.
7. Wash 3X10 minutes in aqua dest.
8. Silver enhancement with Aurion R-Gent for 20 minutes at room temperature.
9. Wash 2X5 minutes in aqua dest.
10. Slides were counter stained in Nuclear Fast Red for 45 seconds, dehydrated in ethanol/xylene and mounted with Pertex.

Results and Conclusion

All three methods show expression of FMRP protein in lymphocytes from a healthy individual, whereas no expression is seen in lymphocytes of a fragile X patient.
In lymphocytes, the concentration of FMRP protein is very low, hence utilising alkaline phosphatase a three step immunoincubation is necessary to obtain sufficient reaction product. The alkaline phosphatase protocol has proven to be a reliable test for screening large numbers of male patients for the fragile X syndrome. However, a major disadvantage of this method might be the presence of endogenous alkaline phosphatase activity in blood cells.
The detection methods with the gold conjugates as described here illustrates that the results obtained using this method are highly comparable to those obtained with the alkaline phosphatase method. The three step immunogold labelling procedure yielded strong labelling of normal lymphocytes with 20 minutes silver enhancement. Even the two step immunogold labelling procedure in combination with two silver enhancement incubations results in sufficient reaction product. The discrimination between the nuclear staining and the coloured reaction product is more easy with the immunogold labelling method than with the alkaline phosphatase method. Both methods show no labelling of lymphocytes from patients with the fragile X syndrome, indicating the high specificity of both labelling procedures. In conclusion : the immunogold labelling method using ultra small gold particles followed by silver enhancement is a good alternative for the alkaline phosphatase method, especially in cases whereby tissues or cells are used with high endogenous alkaline phosphatase activity. In addition, the immunogold method is more sensitive and the reaction product is easier to discriminate when a nuclear counterstaining (Haematoxylin) is necessary.

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